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The Vitamin B_6 Biosynthesis Pathway in *Streptococcus pneumoniae* Is Controlled by Pyridoxal 5′-Phosphate and the Transcription Factor PdxR and Has an Impact on Ear Infection

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Vitamin B_6 is an essential cofactor for a large number of enzymes in both prokaryotes and eukaryotes. In this study, we characterized the pyridoxal 5′-phosphate (PLP) biosynthesis pathway in *Streptococcus pneumoniae*. Our results revealed that *S. pneumoniae* possesses a *de novo* vitamin B_6 biosynthesis pathway encoded by the *pdxST* genes. Purified PdxS functionally displayed as PLP synthase, whereas PdxT exhibited glutaminase activity *in vitro*. Deletion of *pdxS*, but not *pdxT*, resulted in a vitamin B_6 auxotrophic mutant. The defective growth of the Δ*pdxS* mutant in a vitamin B_6-depleted medium could be chemically restored in the presence of the B_6 vitamers at optimal concentrations. By analyzing PdxS expression levels, we demonstrated that the expression of *pdxS* was repressed by PLP and activated by a transcription factor, PdxR. A pneumococcal Δ*pdxR* mutant also exhibited as a vitamin B_6 auxotroph. In addition, we found that disruption of the vitamin B_6 biosynthesis pathway in *S. pneumoniae* caused a significant attenuation in a chinchilla middle ear infection model and a minor attenuation in a mouse pneumonia model, indicating that the impact of vitamin B_6 synthesis on virulence depends upon the bacterial infection niche.

Vitamin B_6 represents the most versatile organic cofactor of enzymes in all organisms. It refers to six biologically interconvertible compounds or vitamers: pyridoxine (PN), pyridoxamine (PM), pyridoxal (PL), and their respective phosphorylated forms, including pyridoxine 5′-phosphate (PNP), pyridoxamine 5′-phosphate (PMP), and pyridoxal 5′-phosphate (PLP) (1). PLP is considered as the only active form of vitamin B_6. In addition to its role in several enzymatic processes, including amino acid and fatty acid metabolism (2, 3), vitamin B_6 is also involved in oxidative stress response and acts to quench reactive oxygen species (4).

Bacteria synthesize PLP via two major pathways: a *de novo* pathway and a salvage pathway (5–7). There are two distinct *de novo* pathways, either deoxyxylulose 5-phosphate (DXP)-dependent or DXP-independent, in different organisms (5–7). The genes of the DXP-dependent pathway have been identified mostly in the γ subdivision of proteobacteria, which form a tight cluster around *pdxA* and *pdxJ* of *Escherichia coli* (5, 6). In this pathway, the biosynthetic product PNP is oxidized by PdxH to produce PLP (8). The DXP-independent pathway (or alternative pathway) requires two enzymes, PdxS (also named as Pdx1, SnzP, or YaaD) and PdxT (also referred to Pdx2, SnoP, or YaaE), which are found in all archaea, fungi, plants, and most bacteria (5). PdxS and PdxT directly produce PLP from a pent(μl)ose (ribose 5-phosphate or ribulose 5-phosphate) and a triose (glyceraldehyde 3-phosphate or dihydroxyacetone phosphate) in the presence of glutamine. Biochemical and structural studies revealed that PdxS and PdxT of *Bacillus subtilis* form a hetero-oligomeric complex (9–11).

In addition to the *de novo* pathway, bacteria are also able to synthesize PLP by a salvage pathway (12), in which PLP is synthesized from other B6 vitamers. In this pathway, the vitamers PL, PN, and PM are phosphorylated by a kinase, such as PdxK or PdxY (13). PMP and PNP are then oxidized to PLP by PdxH (14, 15).

The DXP-dependent and the DXP-independent biosynthesis pathways have been mainly established in *E. coli* and *B. subtilis*, respectively. However, these pathways have not been efficiently explored in pathogenic bacteria. In *Helicobacter pylori*, it was reported that the DXP-dependent vitamin B_6 biosynthesis pathway plays a role in optimal bacterial growth, chronic colonization of mice, glycosylated flagellum synthesis, and flagellum-based motility (16). The DXP-dependent vitamin B_6 biosynthesis pathway of *Mycobacterium tuberculosis* is essential for its survival and virulence (17).

Little is known about how the vitamin B_6 biosynthesis pathways are regulated. A recent study displayed that expression of *pdxST* in *Corynebacterium glutamicum* is significantly reduced in the presence of PL and PLP (18). In this bacterium, a MocR-type transcription factor, PdxR, positively controls the transcription of PLP synthase, whereas the *in vitro* binding of PdxR to the *pdxR-pdxST* intergenic DNA is not significantly affected by the addition of B_6 vitamers (18).

The vitamin B_6 biosynthesis pathway has not been characterized in *Streptococcus pneumoniae*, a Gram-positive bacterium responsible for several human diseases, including pneumonia, septicemia, otitis media (OM), bacteremia, sinusitis, and meningitis. In the present study, we characterized the genes encoding the vitamin B_6 biosynthesis pathway in *S. pneumoniae* using both genetic and biochemical approaches.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
S. pneumoniae		
D39	*S. pneumoniae* serotype 2	40
ST588	D39 Δ*cbpA*::Janus cassette; Kan^r	22
ST606	OM strain of *S. pneumoniae;* Strep^r	21
ST1809	ST606 Δ*arcT*::Janus cassette; Kan^r	This study
ST2675	D39 Δ*pdxT*::Janus cassette; Kan^r	This study
ST2676	D39 Δ*pdxS*::Janus cassette; Kan^r	This study
ST2677	D39 Δ*pdxST*::Janus cassette; Kan^r	This study
ST2726	D39 Δ*pdxR*::Janus cassette; Kan^r	This study
ST2784	ST2676(pST2782); Kan^r Erm^r	This study
ST2785	ST2676(pVA838); Kan^r Erm^r	This study
ST2786	ST2726(pST2783); Kan^r Erm^r	This study
ST2787	ST2726(pVA838); Kan^r Erm^r	This study
ST2790	ST606 Δ*pdxS*::Janus cassette; Kan^r	This study
ST2792	ST2790(pST2782); Kan^r Erm^r	This study
ST2793	ST2790(pVA838); Kan^r Erm^r	This study
E. coli		
DH5α	*E. coli* strain used for cloning	Lab stock
BL21(DE3)	*E. coli* strain used for expression	Novagen
ST2699	*E. coli* BL21(DE3)(pST2697); Kan^r	This study
ST2700	*E. coli* BL21(DE3)(pST2698); Kan^r	This study
ST2728	*E. coli* BL21(DE3)(pST2727); Kan^r	This study
ec022	*E. coli* BW25113 (parent strain for the Keio Collection)	25, 29
ec048	JW0051-1(*pdxA*764:*kan*); Kan^r	25
ec053	JW2548-1(*pdxJ*736:*kan*); Kan^r	25
ec061	ec048(pGB104); Ap^r	This study
ec065	ec053(pGB104); Ap^r	This study
ST2683	*E. coli* ec048(pST2679); Ap^r	This study
ST2684	*E. coli* ec053(pST2679); Ap^r	This study
ST2688	*E. coli* ec048(pST2681); Ap^r	This study
ST2689	*E. coli* ec053(pST2681); Ap^r	This study
Plasmids		
pET28a(+)	His tag expression vector; Kan^r	Novagen
pST2697	pET28a(+) carrying *S. pneumoniae pdxS* ORF; Kan^r	This study
pST2698	pET28a(+) carrying *S. pneumoniae pdxT* ORF; Kan^r	This study
pST2727	pET28a(+) carrying *S. pneumoniae* SPD_1225 ORF; Kan^r	This study
pGB104	pBR322 with *E. coli crp* promoter; Ap^r	This study
pST2679	pGB104 carrying *S. pneumoniae pdxS* ORF; Ap^r	This study
pST2681	pGB104 carrying *S. pneumoniae pdxT* ORF; Ap^r	This study
pVA838	*S. pneumoniae* expression vector; Erm^r	23
pST2782	pVA838 carrying *S. pneumoniae pdxS* promoter and ORF; Erm^r	This study
pST2783	pVA838 carrying *S. pneumoniae pdxR* promoter and ORF; Erm^r	This study

[a] Erm^r, erythromycin resistance; Ap^r, ampicillin resistance; Strep^r, streptomycin resistance; Kan^r, kanamycin resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the bacterial strains used in the present study are listed in Table 1. *S. pneumoniae* D39 (serotype 2, ATCC) and its derivatives were initially grown in Todd Hewitt broth containing 0.5% yeast extract (THY) (BD Biosciences), and all of the working stocks for the present study were grown in a chemically defined medium (CDM; purchased from JRH Bioscience) supplemented with 0.1% choline, 0.25% sodium bicarbonate, and 0.073% cysteine, except as specified. A formulated CDM (complete CDM) was prepared as reported (19), which contains 1 mg of both PM and PL/liter. When necessary, both PM and PL were dropped out (designated as depleted CDM), and the depleted CDM was then supplemented with various concentrations of B6 vitamers, as specified (supplemented CDM). Competence THY medium was prepared by adding 0.2% glucose, 0.2% $CaCl_2$, and 0.02% bovine serum albumin to THY medium (pH 7.2 to 7.4), which was used for transformation of pneumococcal strains (20). If necessary, 200 μg of kanamycin/ml, 2 μg of erythromycin/ml, or 150 μg of streptomycin/ml was used for selection. All pneumococcal strains were routinely grown at 37°C with 5% CO_2. *E. coli* strains were grown in either Luria-Bertani (LB) or M9 minimal medium at 37°C. If necessary, 25 μg of kanamycin/ml, 100 μg of ampicillin/ml or 200 μg of erythromycin/ml was used for selection.

Construction of mutants and complementation in *S. pneumoniae*. All of the primers used in the present study are listed in Table S1 in the supplemental material. *pdxS*, *pdxT*, *pdxST*, and *pdxR* mutants were generated by homologous recombination similarly, as reported earlier (21, 22). Briefly, the upstream and the downstream flanking regions for each target gene were PCR amplified from either D39 or ST606 genomic DNA. The products were digested and ligated with a Janus cassette, which contains a kanamycin resistance gene and was amplified from ST588 (22). The ligated fragments were then used to transform D39 or ST606. The mutants were selected with kanamycin and verified by PCR. For complementation of the Δ*pdxS* and the Δ*pdxR* mutants, the putative promoter and the open reading frame (ORF) of the respective gene was cloned in the pVA838 plasmid (23) and was transformed into the mutant. As a control, pVA838 was also transformed into both the mutants. The transformants were selected with erythromycin and verified by PCR.

Morphological study. *S. pneumoniae* D39 wild-type (WT) and its derivatives were grown in complete CDM to an optical density at 620 nm (OD_{620}) of 0.6. Bacteria were thoroughly washed with depleted CDM and then incubated in either supplemented CDM with 10 μM PLP or depleted CDM for 4 h. The bacterial morphologies were observed under a light microscope after Gram staining (Fisher Diagnostics).

Heterogeneous expression of *S. pneumoniae pdxS* and *pdxT* in *E. coli* Δ*pdxA* and Δ*pdxJ*, respectively. An *E. coli crp* promoter was amplified by PCR from DH5α genomic DNA and was cloned into pBR322 to generate pGB104. The flanking region is similar to that in pMBC664 (24), except that different restriction sites were introduced. ORFs of *S. pneumoniae pdxS* and *pdxT* were then PCR amplified and cloned into pGB104. These constructs, along with pGB104, were individually transformed into a Δ*pdxA* strain JW0051-1 (CGSC8361) and a Δ*pdxJ* strain JW2548-1 (CGSC10027) of *E. coli* (25). The recombinant strains were grown in M9 medium in the presence or absence of 0.1 mM PLP.

Protein expression and purification. ORFs of *S. pneumoniae pdxS*, *pdxT*, and *pdxR* were PCR amplified from D39 genomic DNA. The PCR products were cloned into pET28a(+) (Novagen) at the same restriction sites as indicated in the respective primers (see Table S1 in the supplemental material). The recombinant plasmids were verified by sequencing and used to transform *E. coli* BL21(DE3). The expression of each recombinant strain was induced for 3 h at 22°C with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) in LB broth. Another aliquot of recombinant bacteria that expresses PdxR was washed with phosphate-buffered saline (PBS) and then resuspended in M9 broth for induction with 0.5 mM IPTG. Protein purification was performed as described previously using Ni-NTA resin (Qiagen) (26, 27). The purified proteins were dialyzed against PBS with 10% glycerol and stored at −80°C until use.

Glutaminase activity. Glutaminase activity was determined using a procedure as described previously (10). Briefly, 10 μM PdxT, 10 μM PdxS, or a 10 μM mixture of both proteins was incubated with 1 U of L-glutamic dehydrogenase from bovine liver (Sigma), 10 mM glutamine, 0.6 mM 3-acetylpyridine adenine dinucleotide (APAD; Sigma). After 1 h of preincubation at 37°C, the OD was measured every 10 min at 363 nm (OD_{363}) over an 80-min period.

PLP formation assay. PLP formation was analyzed according to an earlier report (10). Briefly, 10 μM PdxS, 10 μM PdxT, or a 10 μM mixture of both proteins was incubated in the presence of 10 mM glutamine or 10 mM NH_4Cl, as specified, 1 mM D-ribose 5-phosphate, 2 mM DL-glyceraldehyde 3-phosphate, and 50 mM Tris-HCl (pH 8.0) in a final volume of 500 μl. After 30 min of preincubation at 37°C, the OD at 414 nm (OD_{414}) was measured every 10 min for a total of 3 h.

Electrophoretic mobility shift assay (EMSA). A ^{32}P-end-labeled *pdxS* probe (0.01 pmol) was used in each 10-μl binding reaction mixture as described previously (26, 28), with modifications. Briefly, purified His-PdxR (at specified concentrations) and DNA probes were incubated for 30 min at room temperature in DNA binding buffer, followed by electrophoresis on a nondenaturing 8% polyacrylamide gel for 2 to 3 h at 14 V/cm in 0.5× Tris-borate-EDTA (TBE) buffer. A 500-fold excess of unlabeled DNA fragments was used for competition experiments. Gels were vacuum dried, exposed on a phosphor screen, scanned with a Storm 860 PhosphorImager (Molecular Dynamics), and analyzed with ImageQuant software.

RNA preparation and Northern blot analysis. Wild-type (WT) D39 was grown in 10 ml of complete CDM to an OD_{620} of 0.6 and then split into two 5-ml aliquots. The bacteria were washed three times with depleted CDM and then resuspended in 5 ml of either depleted CDM or supplemented CDM with 10 μM PLP. After 4 h of incubation at 37°C with 5% CO_2, RNA samples were prepared from these bacteria using an RNeasy minikit (Qiagen). Then, 10 μg of total RNA of each sample was separated on a 1% agarose gel with 1× morpholinepropanesulfonic acid buffer and 2% formaldehyde. Subsequently, the RNA was transferred overnight by capillarity to a Hybond membrane using 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as the transfer buffer.

Hybridization with a [γ-^{32}P]ATP (MP Biomedicals)-labeled oligonucleotide specific to *pdxS* was performed in hybridization buffer (GE Healthcare) for 5 h at 42°C. The membrane was washed twice with a washing buffer (2× SSC, 0.1% sodium dodecyl sulfate [SDS]), exposed overnight to a phosphor screen, and visualized using a PhosphorImager (Molecular Dynamics). Hybridization with a probe specific to *S. pneumoniae* 16S rRNA was used as a loading control.

Ethics statement. This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee of Albany Medical College (permits 11-12002 for mice and 11-12001 for chinchillas). All efforts were made to minimize suffering.

Preparation of polyclonal antibodies and Western blot analyses. Female BALB/c mice (Taconic) were immunized subcutaneously to generate polyclonal antibodies against purified PdxS and PdxT, respectively ($n = 5$). The procedure of immunization and antisera preparation were similar to those described earlier (27).

For Western blot analyses, bacteria were grown in complete CDM to an OD_{620} of 0.6 and treated as described in the "morphological study" section above. After 2 to 4 h of incubation, bacteria were broken by sonication, and equal amounts of proteins from each sample were loaded onto a SDS–12.5% PAGE gel. Separated proteins were transferred to a polyvinylidene difluoride membrane and probed with the antisera to PdxT or PdxS. The analysis with a polyclonal antibody against SPD_1063 was used as a loading control. After blotting with a peroxidase-conjugated goat anti-mouse IgG secondary antibody (Thermo Scientific), peroxidase detection was carried out with an ECL Western blotting detection reagents and analysis system (Thermo Scientific).

Mouse infection. We infected 6- to 8-week-old female BALB/c mice (Taconic) with WT, ST2675, and ST2676 strains ($n = 8$). Briefly, bacteria were grown to an OD_{620} of 0.4 in complete CDM, washed four times in PBS, diluted with PBS to ~1×10^8 CFU/ml, and then inoculated intranasally in a volume of 50 μl (~5×10^6 CFU). The inocula were determined by serial dilution and plate counting. Mice were subsequently monitored for 9 days.

Coinfection of chinchilla middle ears. Coinfection experiments were carried out as described previously (21) with slight modifications. Three 1-year-old female chinchillas (Ryerson Chinchilla Ranch) were used in each group. Broth cultures of WT (ST606) and its isogenic mutants were separately incubated to an OD_{620} of 0.4 and stocked with 25% glycerol in THY. The CFU levels of the frozen stocks were enumerated for WT in the presence of streptomycin or for mutant in the presence of kanamycin. The bacteria were diluted and mixed in PBS at a 1:1 ratio of CFU. Aliquots of 100-μl mixtures were used to infect the middle ears of chinchillas (~1×10^4 CFU/ear, $n = 6$). The level of attenuation is expressed as the competitive index (CI), which is defined as the output CFU ratio (mutant/WT) divided by the input CFU ratio (mutant/WT). In addition, ST1809, a Δ*arcT* mutant that did not affect the bacterial virulence in our previous experiment (unpublished) was used as a control in this experiment.

Statistical analysis. Comparisons of growth were analyzed using a two-tailed *t* test. Kaplan-Meier survival curves were compared using Gehan-Breslow-Wilcoxon test. All of the analyses were performed using Prism 5 (GraphPad Software), and *P* values of < 0.05 were considered to be statistically significant.

RESULTS

***S. pneumoniae* PdxS and PdxT are the enzymes for PLP synthesis.** We used the *S. pneumoniae* D39 strain as a model organism to study the biosynthesis pathway of vitamin B_6. In this strain, SPD_1297 (*pdxS*) has been annotated as a putative pyridoxal biosynthesis lyase, and SPD_1296 (*pdxT*) encodes a putative glutamine amidotransferase subunit (30). The two genes are clustered in a putative operon (Fig. 1A). Both PdxS and PdxT share high identity (64 and 44%, respectively) in the amino acid sequences with their orthologs in *B. subtilis*. In the present study, *S. pneumoniae pdxS* and *pdxT* were overexpressed in *E. coli*, and the recombinant proteins were purified to high homogeneity using Ni-NTA chromatography. These proteins were then used to determine their activities in vitamin B_6 formation *in vitro*.

To test the glutaminase activity, pneumococcal PdxT was incubated individually or together with PdxS using glutamine as a substrate. The activity was exclusively detected when both proteins were present in the reaction but not with either PdxT or PdxS alone (Fig. 1B). This result indicates that the glutaminase activity detected with PdxT is dependent on the presence of PdxS. This finding coincides with previous observations in other organisms (17, 31–35).

Glutamine, glyceraldehyde 3-phosphate, and ribose 5-phosphate were used as substrates to determine the PLP synthesis activity displayed by pneumococcal PdxS, PdxT, or a mixture of the two proteins. As a result, the PLP synthase activity was detected only when both PdxS and PdxT were concomitantly present in the reaction, whereas no PLP was detectable with either individual protein (Fig. 1C). This result indicates that either the interaction between PdxS and PdxT is required for the PLP formation or the formation of PLP is a sequential reaction in which the product of one enzyme is used as a substrate for the other enzyme.

In *B. subtilis*, PdxT and PdxS form an active complex, which is required for PLP biosynthesis (9–11, 31). In our study, the glutaminase and the PLP synthase activities were exclusively detected in the presence of both proteins. Therefore, we examined the interaction between *S. pneumoniae* PdxS and PdxT *in vitro*, either under the same conditions as described by others (10, 31) or using conditions in which glutaminase activity or PLP synthase activity was detected. The proteins were then separated on a native gel and detected either by staining with Coomassie brilliant blue or by Western blotting analyses with both anti-PdxS and anti-PdxT antibodies. Our result showed that the mobility of PdxS and PdxT when mixed was identical to that of the two proteins analyzed singularly (data not shown). Thus, *S. pneumoniae* PdxS and PdxT do not appear to form a complex under these conditions.

***S. pneumoniae* Δ*pdxS* is auxotrophic for vitamin B_6.** In *S. pneumoniae*, *pdxS* and *pdxT* genes are immediately adjacent to

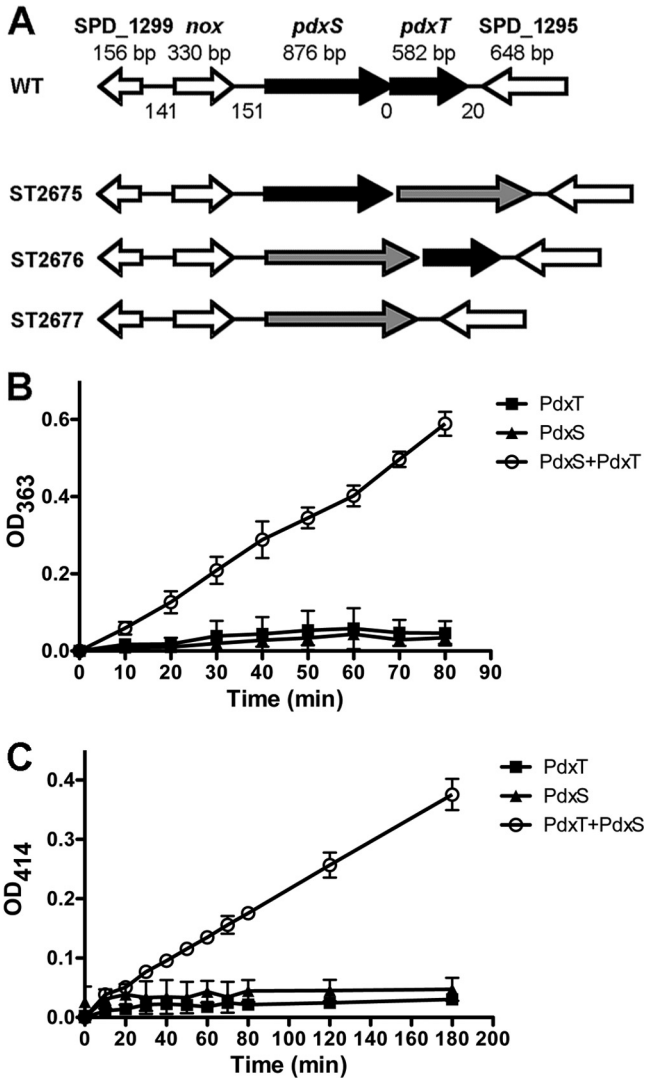

FIG 1 Enzymatic activity assays of PdxS and PdxT. (A) Genetic organization of *pdxS* and *pdxT* in *S. pneumoniae*. The size (in base pairs) of each ORF is indicated above the gene. The size of each intergenic region is also noted between adjacent genes. Each target gene was replaced with a Janus cassette (gray arrow) by homologous recombination. (B and C) Glutaminase activity (B) or PLP synthase activity (C) was determined in the presence of PdxS, PdxT, or a mixture of the two proteins at a molar ratio of 1:1. The data shown are the means of three independent assays. Error bars denote the standard error of the mean (SEM).

each other (Fig. 1A). To study the pneumococcal vitamin B_6 biosynthesis pathway *in vivo*, we replaced *pdxS*, *pdxT*, and *pdxST* with a Janus cassette and designated these strains ST2676, ST2675, and ST2677, respectively (Fig. 1A). These mutants were verified by Western blotting with specific antibodies (see Fig. S1 in the supplemental material). Interestingly, PdxT was still detected in ST2676, although *pdxS* and *pdxT* are clustered in a putative operon (Fig. 1A). The expression of *pdxT* in ST2676 is likely controlled by a promoter from the inserted Janus cassette.

The growth of these mutants was determined in complete CDM, which contains PM and PN, and in depleted CDM which does not have any B6 vitamer. The growth rates of all of the mutants were indistinguishable from WT grown in complete CDM (data not shown). In depleted CDM, ST2675 was able to grow, but its growth rate was lower than that of the WT. In contrast, ST2676 and ST2677 were unable to grow in depleted CDM (Fig. 2A), suggesting that the Δ*pdxS* mutant is auxotrophic for vitamin B_6. Since PdxT was detected in ST2676 (see Fig. S1 in the supplemental material), the vitamin B_6 auxotrophy of this strain is mainly caused by the absence of PdxS, which is consistent with the observation that expression of *pdxS* alone in both ST2676 and ST2677 partially rescued the bacterial growth in depleted CDM (data not shown).

The defective growth of the Δ*pdxS*, Δ*pdxT*, and Δ*pdxST* mutants in depleted CDM was restored by exogenous PLP in a concentration-dependent manner (Fig. 2A). In contrast, addition of up to 10 μM PLP exhibited no effect on the growth of WT (Fig. 2A). Surprisingly, 100 μM PLP was toxic to both WT and ST2676 (Fig. 2B). Therefore, PLP in *S. pneumoniae* needs to be maintained at precise levels.

***S. pneumoniae* has a functional salvage pathway.** Most bacteria have a salvage pathway for vitamin B_6 biosynthesis that enables them to utilize other B6 vitamers for PLP synthesis. However, PdxH and PdxK, which catalyze the conversions between different B_6 vitamers, could not be found in the genome of D39 (30). To determine whether *S. pneumoniae* has a functional salvage pathway, WT and ST2676 were grown in supplemented CDM which was provided with different concentrations of PL, PM, or PN. In the presence of 1 or 10 μM each tested vitamer, the growth of the WT was not affected, but the growth of ST2676 was partially (at 1 μM PN) or fully (at 1 μM PL or PM or 10 μM any of the vitamers) restored to the WT levels (Fig. 2B). This result suggests that a functional savage pathway exists in *S. pneumoniae*.

Morphologies of the pneumococcal *pdx* mutants. To analyze the effects of vitamin B_6 biosynthesis on the bacterial morphology, we performed Gram-staining to compare ST2675, ST2676, and ST2677 with the WT strain. These bacterial strains were first grown in complete CDM and then incubated in either depleted CDM or supplemented CDM with 10 μM PLP. Our result showed that the chain sizes of all of the strains were similar in the presence of PLP. In the absence of PLP, both WT and ST2675 exhibited similar chain sizes comparable to that in the presence of PLP, whereas ST2676 and ST2677 appeared as mono- or diplococci (Fig. 2C). The morphological changes of ST2676 and ST2677 in the absence of PLP are consistent with their growth phenotypes in depleted CDM.

The activity of *S. pneumoniae* PdxS is independent of PdxT in the presence of ammonium. It has been shown that the addition of exogenous ammonium in the growth medium compensates the defective growth rate of a Δ*pdxT* mutant in *B. subtilis* (31), suggesting that the primary role of PdxT in vitamin B_6 synthesis is providing ammonium. In the present study, we grew WT, ST2675, ST2676, and ST2677 in depleted CDM, but supplemented with various concentrations of ammonium. The result showed that the defective growth of ST2675 was partially (with 0.1 or 1 mM ammonium) or fully (with 10 mM ammonium) restored to the WT levels with additional ammonium (Fig. 3A). In contrast, the growth of neither WT nor ST2676 or ST2677 was changed in the presence of 0.1 to 10 mM ammonium. These results indicate that the primary function of pneumococcal PdxT is similar to its ortholog in *B. subtilis*.

We showed that both PdxS and PdxT were required to synthesize PLP *in vitro* (Fig. 1C). Based upon the ammonium-dependent

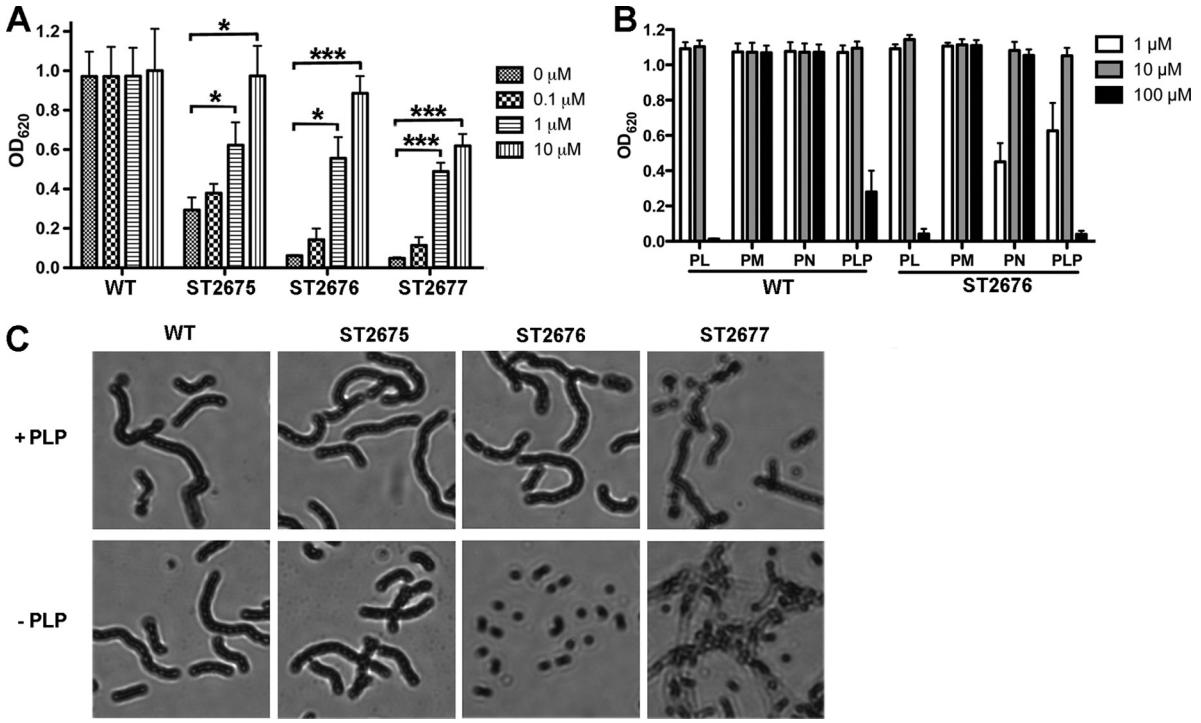

FIG 2 Growth phenotypes of the *S. pneumoniae* mutants associated with the vitamin B_6 biosynthesis pathway. (A) Growth of WT (D39) and its derivatives, including Δ*pdxT* (ST2675), Δ*pdxS* (ST2676), and Δ*pdxST* (ST2677), in depleted CDM (0 μM) or supplemented CDM with indicated concentrations of PLP. Bacteria were grown for 8 h at 37°C with 5% CO_2. The data shown are the means of three independent experiments. Error bars denote the SEM. *, $P < 0.05$; ***, $P < 0.001$. (B) Growth of WT (D39) and ST2676 in supplemented CDM with various concentrations of indicated vitamers for 8 h at 37°C with 5% CO_2. The data shown are the means of three independent experiments. Error bars denote the SEM. (C) Gram staining result of WT (D39) and its derivatives after incubation in supplemented CDM with 10 μM PLP (+ PLP) or in depleted CDM (− PLP).

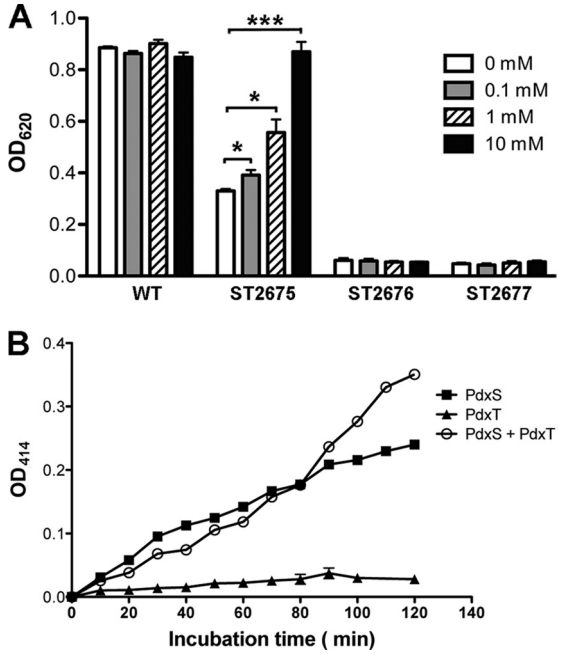

FIG 3 PLP synthase activity of *S. pneumoniae* PdxS. (A) Growth of WT (D39) and its derivatives, including Δ*pdxT* (ST2675), Δ*pdxS* (ST2676), and Δ*pdxST* (ST2677) strains, in depleted CDM (0 mM) or supplemented CDM with various concentrations of NH_4Cl for 8 h at 37°C with 5% CO_2. The data shown are the means of three independent experiments. Error bars denote SEM. *, $P < 0.05$; ***, $P < 0.001$. (B) PLP formation assay in the presence of PdxS, PdxT, or a mixture of the two proteins at a molar ratio of 1:1. The data shown are the means of triplicate samples. Error bars denote the SEM.

growth phenotype of ST2675, we hypothesized that the function of PdxT in this reaction was producing ammonium from glutamine. Therefore, replacing glutamine with ammonium in the reaction might allow PdxS to produce PLP in the absence of PdxT. We tested this hypothesis *in vitro*, and the result showed that PdxS alone could form PLP after replacing glutamine with ammonium in the reaction (Fig. 3B). These results are consistent with a two-step model for PLP formation, in which PdxT and PdxS together convert glutamine into ammonium, and PdxS utilizes ammonium, glyceraldehyde 3-phosphate (or dihydroxyacetone phosphate), and ribose 5-phosphate (or ribose 5-phosphate) to produce PLP (9, 11).

It has been reported that *Cercospora nicotianae pdx1* complements an *E. coli pdxJ* mutation (36). We also investigated whether heterogeneous expression of *S. pneumoniae pdxS or pdxT* could complement *E. coli* mutants defective in vitamin B_6 biosynthesis. The ORFs of both *pdxS* and *pdxT* were cloned into plasmid pGB104 to express these genes under the control of the *E. coli crp* promoter. These constructs and the control plasmid pGB104 were then individually transformed into *E. coli* Δ*pdxA* (ec048) or Δ*pdxJ* (ec053). These mutants and their derivatives were compared to the WT strain (ec022) for growth either in M9 broth (Fig. 4A and B) or on M9 plates (see Fig. S2 in the supplemental material), in the presence or absence of 0.1 mM PLP or PN. The results showed that neither ec048 nor ec053 grew with the minimal media in the absence of B_6 vitamers, but both the mutants grew similarly to their parental strains by the addition of exogenous PN or PLP. In the absence of B_6 vitamers, the growth defect of both the mutants

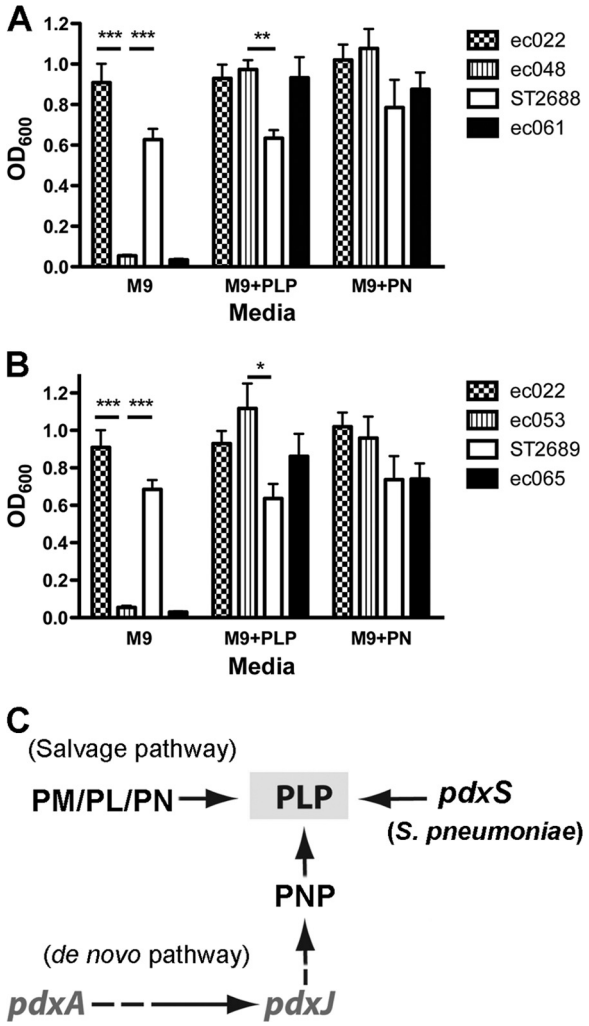

FIG 4 Heterogeneous expression of *S. pneumoniae pdxS* in the *E. coli* Δ*pdxA* and Δ*pdxJ* mutants. (A and B) Complementation of *E. coli* Δ*pdxA* (A) or Δ*pdxJ* (B) mutants by expression of *S. pneumoniae pdxS*. ec022 is the *E. coli* WT strain. ec048 and ec053 are *E. coli* Δ*pdxA* and Δ*pdxJ*, respectively. ST2688 and ST2689 express pneumococcal *pdxS* in Δ*pdxA* and Δ*pdxJ*, respectively. Strains ec061 and ec065 contain the control vector in Δ*pdxA* and Δ*pdxJ*, respectively. Bacteria were grown in M9 broth in the absence (M9) or presence of either 0.1 mM PLP (M9 + PLP) or 0.1 mM PN (M9 + PN) for 8 h shaking at 37°C. The data shown are the means of three independent experiments. Error bars denote the SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C) Schematic representation of *E. coli* vitamin B_6 biosynthesis pathways and the heterogeneous expression of *S. pneumoniae pdxS* in the *E. coli* mutants.

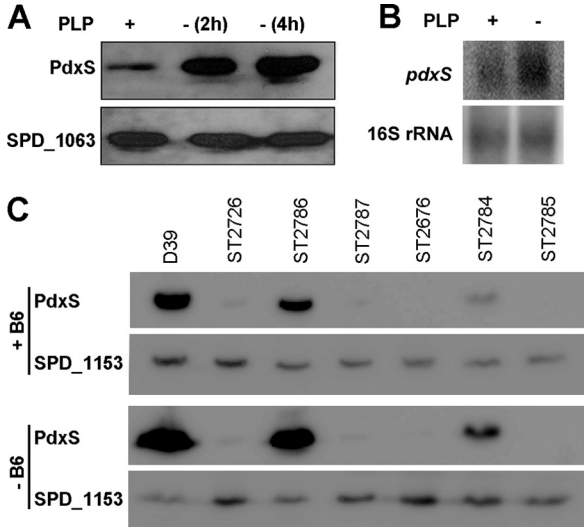

FIG 5 Regulation of *S. pneumoniae pdxS*. (A) Western blot analysis of WT (D39) incubated in either supplemented CDM with 10 μM PLP (+) or depleted CDM (−) for 2 or 4 h. The protein samples were analyzed with the indicated antibodies (right side). Blotting with antibody against SPD_1063 was used as a loading control. (B) Northern blot of total RNA samples isolated from WT (D39) incubated in either supplemented CDM with 10 μM PLP (+) or depleted CDM (−) for 4 h. The blot with the16S rRNA probe was used as a loading control. (C) Expression of PdxS in strains as indicated, including WT (D39), ST2726 (Δ*pdxR*), ST2786 (Δ*pdxR*/*pdxR*+), ST2787 (Δ*pdxR*/vector control), ST2676 (Δ*pdxS*), ST2784 (Δ*pdxS*/*pdxS*+), and ST2785 (Δ*pdxS*/vector control). Bacteria were grown in CDM with vitamin B_6 (+B6) and an aliquot of each strain was washed and incubated with depleted CDM (−B6). Bacteria were subsequently disrupted by sonication, and protein samples were detected by Western blotting analyses with the anti-PdxS antibody. An antibody against SPD_1153 was used as a loading control.

could be restored by the expression of pneumococcal *pdxS*, but not by the control plasmid (Fig. 4A and B) or expression of *pdxT* (data not shown). This result also suggests that pneumococcal PdxS is capable of producing PLP independent of PdxT in the presence of ammonium (Fig. 4C).

The expression levels of PdxS are feedback inhibited by PLP. The vitamin B_6 biosynthesis pathways have been studied in several bacteria, but the knowledge regarding regulation of the PLP levels remains very limited. We grew *S. pneumoniae* WT in complete CDM and then incubated the bacteria in either depleted CDM or supplemented CDM with 10 μM PLP. The expression levels of PdxS were then determined by Western blotting with antibody against pneumococcal PdxS. Our result showed that PdxS was weakly expressed in CDM supplemented with PLP, but it was abundant after incubation for 2 to 4 h in the absence of PLP (Fig. 5A), suggesting a feedback inhibition of vitamin B_6 biosynthesis by PLP.

We further determined the expression of *pdxS* using Northern blotting. The result showed that *pdxS* mRNA levels were enhanced in the absence PLP compared to that in the presence of PLP (Fig. 5B), which is consistent with the result of Western blot analysis. Taken together, it is likely that the expression of *pdxS* is transcriptionally regulated.

Expression of *pdxS* is directly activated by PdxR. In *C. glutamicum*, it has been reported that the *pdxST* operon is transcriptionally regulated by a transcription factor PdxR (18), which belongs to the MocR subfamily of the GntR-type proteins. PdxR carries a winged helix-turn-helix (wHTH) domain at the N-terminal portion and an aspartate aminotransferase-like (AAT) domain at the C-terminal portion with potential PLP binding sites (18). In *S. pneumoniae*, we identified five genes each having a wHTH domain of the GntR superfamily (see Fig. S3A in the supplemental material). However, only SPD_1225 possesses an AAT domain and shares all of the characteristics with *C. glutamicum* PdxR (see Fig. S3A in the supplemental material), and the two proteins only share 21% identity in amino acid sequences. In addition, *C. glutamicum pdxR* is adjacent to *pdxS*, whereas *S. pneumoniae* SPD_1225 is over 70 genes away from *pdxS*. We designated the SPD_1225 gene *pdxR* and the encoded protein PdxR hereafter unless specified. To investigate the role of *S. pneumoniae*

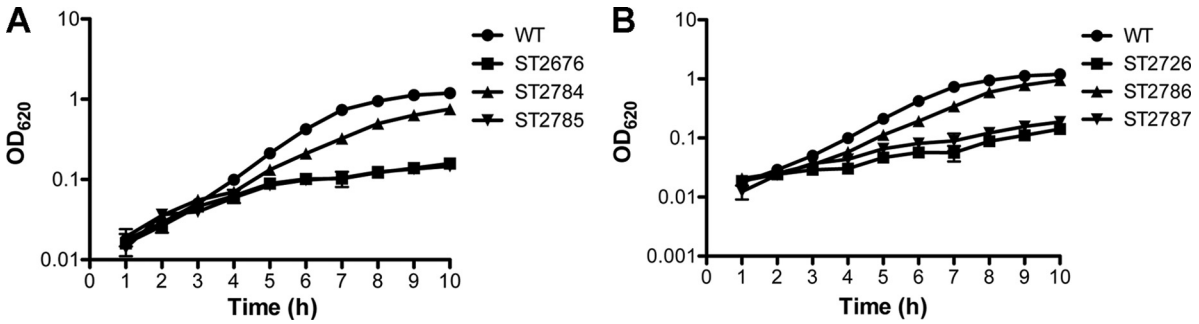

FIG 6 Growth of *S. pneumoniae* Δ*pdxS* (A) and Δ*pdxR* (B) in depleted CDM. The bacterial strains tested include WT (D39), ST2676 (Δ*pdxS*), ST2784 (Δ*pdxS*/*pdxS*+), ST2785 (Δ*pdxS*/vector control), ST2726 (Δ*pdxR*), ST2786 (Δ*pdxR*/*pdxR*+), and ST2787 (Δ*pdxR*/vector control). Bacterial growth was monitored hourly at OD_{620}. The data shown are the means of three repeat experiments. Error bars denote the SEM. Note that ST2676 and ST2785 are indistinguishable in panel A.

PdxR in regulation of *pdxS*, we generated a Δ*pdxR* mutant strain (ST2726) and complemented ST2726 with a native *pdxR* under its own promoter (ST2786). The Δ*pdxS* mutant was also complemented with a native *pdxS* (ST2784). The deletion and the complementation of both *pdxS* and *pdxR* were verified by PCR (Fig. 3B). We then explored the expression of PdxS in response to vitamin B_6 with these mutants. Our result showed that the expression of PdxS was almost completely abolished in ST2726 either in the presence or absence of vitamin B_6 (Fig. 5C), indicating that PdxR is an activator of *pdxS*. This phenotype can be corrected by the expression of *pdxR*, but not by the vector control (Fig. 5C), indicating specificity.

We grew ST2676, ST2726, and their complemented strains in either complete CDM or depleted CDM. Our result showed that both the mutants grew similarly to the WT in the presence of vitamin B_6 (see Fig. S4 in the supplemental material) but were fully auxotrophic for vitamin B_6 (Fig. 6). The growth phenotypes of both mutants in the absence of vitamin B_6 can be corrected by complementation with their respective genes but not by the vector control (Fig. 6). These results are consistent with the Western blot analysis data (Fig. 5C).

In order to determine whether the regulation of *pdxS* by PdxR is direct, we purified His-tagged PdxR from recombinant *E. coli* and then performed EMSA with this protein. Our result showed that the mobility of the labeled *pdxS* promoter DNA was retarded in the presence of PdxR (Fig. 7A). The free probe was detected when 500-fold excess of unlabeled *pdxS* DNA was present in the binding reaction (Fig. 7B). These results indicate that the interaction between the PdxR protein and the *pdxS* promoter DNA is specific. In addition, more unbound probe was detected when 1

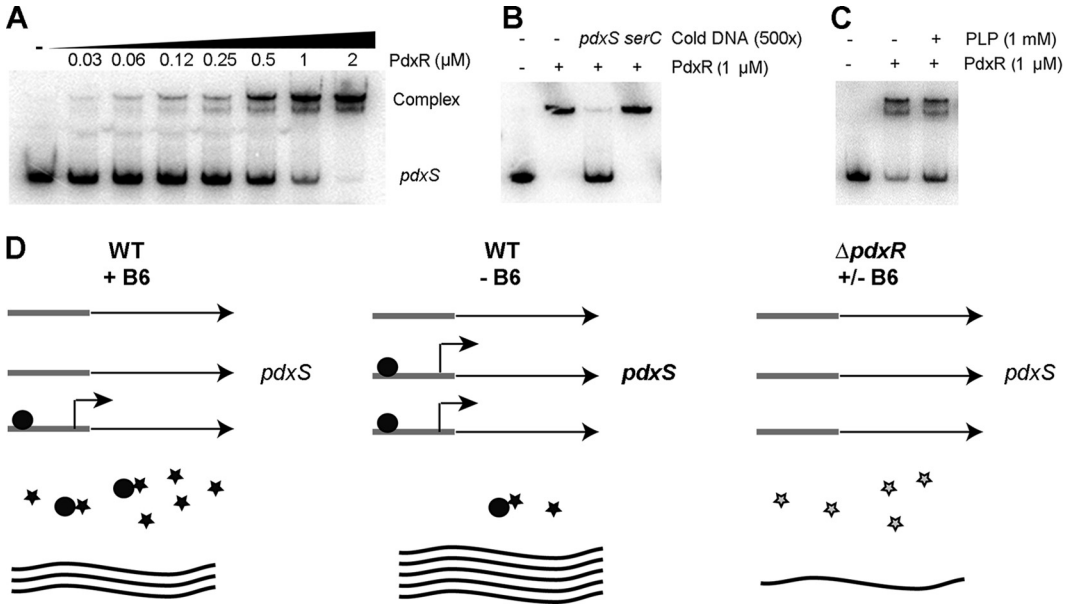

FIG 7 Regulation of *pdxS* expression by PdxR. (A) EMSA experiment showing binding of the purified PdxR protein to the labeled putative *pdxS* promoter DNA. (B) Binding of the *pdxS* probe by PdxR in the presence of the 500-fold excess unlabeled (Cold) *pdxS* promoter DNA. The unlabeled *M. tuberculosis serC* promoter DNA is served as a nonspecific control. (C) Interaction of PdxR with the *pdxS* probe in the presence or absence of 1 mM PLP. This result is a representative of three repeat experiments. (D) Regulation of *pdxS* by PdxR and PLP under different growth conditions. A mechanistic model showing that *pdxS* expression requires the activator PdxR, and the expression levels of *pdxS* in the WT are higher when less PLP is available. In the Δ*pdxR* mutant, *pdxS* was expressed at the baseline levels either in the presence or absence of vitamin B_6. Black ball, PdxR protein; black star, PLP molecule; gray star, with or without PLP; curved line, *pdxS* transcript.

mM PLP was added in the binding reaction (Fig. 7C), indicating that the affinity between PdxR-*pdxS* is reduced in the presence of PLP. We hypothesized that the PdxR protein we applied in the EMSA experiments had bound, at least partially, with PLP when it was purified. To test this hypothesis, we washed the recombinant *E. coli* thoroughly and resuspended the bacteria in M9 broth during induction for expressing PdxR. The protein purified from this procedure enhanced its affinity with *pdxS* promoter DNA by ~2-fold more than that induced in LB broth (data not shown). Based on our data (Figs. 5 to 7), we propose a model for the regulation of *pdxS* by PLP and PdxR (Fig. 7D). At high concentrations of PLP, more PdxR binds PLP. The PLP-bound PdxR has a low affinity with the *pdxS* promoter DNA, thus the expression of *pdxS* is relatively low. However, when environmental PLP is reduced, PdxR-*pdxS* affinity is enhanced, which in turn activates the expression of *pdxS*. In the Δ*pdxR* mutant, *pdxS* is expressed at baseline levels either in the presence of absence of vitamin B_6.

***S. pneumoniae* PdxS impacts on infection in a chinchilla OM model, but not in a mouse pneumonia model.** It has been shown that the DXP-independent vitamin B_6 biosynthesis pathway in *M. tuberculosis* plays an essential role in virulence (17). In the present study, we first determined the role of the pneumococcal vitamin B_6 *de novo* biosynthesis pathway in virulence using a mouse pneumonia model. As a result, only 12% of the mice infected with either WT or ST2675 survived until the end of the experiment with a median survival time of 3 days. However, 50% of the ST2676-infected mice survived for the duration of the experiment with a median survival time of 6.5 days (Fig. 8A). Therefore, ST2676 was slightly attenuated compared to WT, although this difference appears to be statistically insignificant ($P = 0.12$).

We argued that the pneumococcus might encounter different levels of vitamin B_6 at various infection niches. Thus, we also infected chinchilla middle ears using a coinfection model with a Δ*pdxS* mutant (ST2790) generated from a clinical isolate (21). Our result showed that ST2790 was significantly attenuated (Fig. 8B). The attenuation could be partially recovered by the expression of *pdxS* (ST2792) but not by transformation with a vector control (ST2793) (Fig. 8B). The attenuations of both ST2790 and ST2793 are significant compared to the ST1809 control ($P < 0.05$).

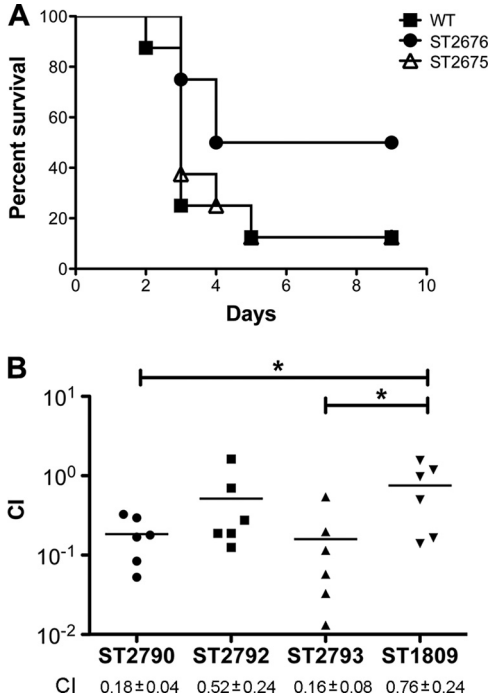

FIG 8 Infection of animals with *S. pneumoniae pdxS* mutants. (A) Survival of infected mice ($n = 8$). Mice were infected intranasally with WT (D39) and its derivatives, including ST2675 (Δ*pdxT*) and ST2676 (Δ*pdxS*), and the survival was monitored for up to 9 days postinoculation. (B) Coinfection of chinchilla middle ears ($n = 6$). Each ear was coinfected with 1:1 mixture of WT (ST606) and its derivatives, including ST2790 (Δ*pdxS*), ST2792 (Δ*pdxS*/*pdxS*+), ST2793 (Δ*pdxS*/vector control), and ST1809 (Δ*arcT*). The bacteria were washed out 3 days postinfection, and the CFU were counted to calculate the competitive index (CI). *, $P < 0.05$.

DISCUSSION

A paradigm of DXP-independent vitamin B_6 biosynthesis pathway includes two enzymes: PdxS and PdxT. This pathway has been well described in *B. subtilis*. In this bacterium, PdxS and PdxT physically form a complex as PLP synthase (9–11, 31, 37), which is also structurally stabilized by glutamine (9–11). The PdxS and PdxT complex is required for glutaminase activity, which converts glutamine into ammonium (9, 11). In addition, a Δ*pdxT* mutant of this bacterium grows well in the presence of sufficient ammonium in the medium (31), supporting that PdxT is not required for PLP synthase if ammonium is available.x

In *S. pneumoniae*, the glutaminase activity of PdxT is also dependent upon the availability of PdxS. However, we failed in detecting a PdxS-PdxT complex when we separated the protein mixture with a native gel. It is possible that the PdxS and PdxT proteins in *S. pneumoniae* differ from their orthologs in *B. subtilis*. We demonstrated that the *S. pneumoniae* Δ*pdxT* strain grew similarly to the WT when sufficient ammonium is available. In addition, the defective growth of the Δ*pdxST* mutant could be partially corrected by complementation with *pdxS* alone (not shown). These observations suggest that PdxS alone is functional to synthesize PLP in the presence ammonium, similar to its ortholog in *B. subtilis*.

In addition to a *de novo* vitamin B_6 biosynthesis pathway, bacteria also have a salvage pathway that converts the B_6 vitamers taken from the environment into PLP. It is interesting that various bacteria differ in usage of these vitamers. Our results showed that *S. pneumoniae* could utilize all of the vitamers we tested including PM, PN, PL, and PLP. However, 100 μM PLP is toxic to this bacterium, whereas *E. coli* grew well with up to 0.5 mM PLP in our study (not shown). When low concentration of a vitamer was supplemented for the growth of the *S. pneumoniae* Δ*pdxS* mutant, bacteria grew preferentially with the vitamers other than PN. This result coincides with what was observed in *B. subtilis* (31, 38). Mutants of the *pdxS* orthologs in *M. tuberculosis* complex bacteria grew well with PN or PL but very poorly with PM or PLP (17; G. Bai, unpublished data). It is likely that these bacteria differ in the uptake of B_6 vitamers or that they are unable to convert certain vitamers to PLP, which warrants further investigation.

PLP has been extensively studied as a cofactor for various enzymes, but its regulatory role is still inadequately explored. A recent study revealed that the DXP-independent pathway in *C. glutamicum* is transcriptionally activated by PdxR and inhibited by PLP (18). However, a link between PdxR and PLP has not been established. In the present study, we demonstrated the same reg-

ulatory effects by PdxR and PLP in *S. pneumoniae*. Furthermore, with the addition of PLP in the *in vitro* reaction, we noticed that the affinity between the PdxR protein and the *pdxS* promoter DNA was reduced, even though it was not dramatic. More interestingly, the recombinant PdxR we induced in M9 broth, free of exogenous B_6 vitamer, enhanced ~2-fold in interaction with the DNA ligand, compared to the protein induced in LB broth (data not shown). These findings are consistent with the observation that a PLP-dependent transcriptional regulator GabR in *B. subtilis* reduces ~2-fold in binding with its DNA ligand by addition of PLP and γ-amino-butyric acid (GABA) (39). Both PdxR and GabR have an AAT domain with putative PLP binding sites. Mutations of the PLP-binding sites in PdxR warrant further investigation to evaluate the role of PLP in the interaction between PdxR and its DNA ligands.

The knowledge pertaining to the vitamin B_6 pathway in pathogenic bacteria is very limited. Recently, it has been shown that the vitamin B_6 biosynthesis is essential for virulence of *M. tuberculosis* (17) and *H. pylori* (16). However, in our mouse infection experiment, the Δ*pdxS* mutant only showed a moderate attenuation and is not statistically significant. It is likely that the levels of vitamin B_6 in the lungs are high enough to compensate the vitamin B_6 defect of ST2676, as an extracellular pathogen. In contrast, the infection niche of *M. tuberculosis* differs from *S. pneumoniae*. *M. tuberculosis* encounters a relatively low vitamin B_6 environment during intracellular replication. This hypothesis was supported by the coinfection experiment that vitamin B_6 synthase gene affects the infection of *S. pneumoniae* in an OM model. Thus, the contribution of vitamin B_6 in bacterial pathogenesis largely depends upon the particular infection niche. Understanding how bacterial metabolism pathways impact on infection will provide a molecular basis antimicrobial therapy.

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